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Understanding the molecular basis of breast carcinoma is central to designing rational and effective therapy for this prevalent disease. We have proposed to investigate several regulatory proteins that we hypothesized may underlie at least a subset of human breast carcinomas. In investigating transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, we have discovered a novel protein, expressed in the breast, that acts as a specific repressor of retinoic acid and thyroid hormone receptors. This 270kDa protein, postulated to mediate retinoic acid and thyroid hormone receptor actions as a repressor of specific gene expression, is recruited to estrogen receptor by antagonists and its regulation appears to underlie restistance in ER positive tumors..

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Michael J Rochelle 8/28/97

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5. SUMMARY OF PROGRESS

Breast cancer is a devastating disease, the etiology of which may be best understood by investigating the regulatory events that can maintain or restore normal growth properties. Our laboratory has investigated control of gene transcription and mammalian development, emphasizing two classes of transcription factors: POU domain factors, and nuclear receptors that mediate both positive and negative patterns of gene expression. Our ongoing studies under this Award are based on the premise that these factors, and trophic receptors under their control, are of specific significance to the etiology and treatment of breast cancer.

We have perused our original goals emphasized transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, and a novel seven-transmembrane helix receptor expressed in breast. Our most important findings have been the discovery of corregulators of estrogen receptors, including novel nuclear receptor co-repressor protein, referred to as N-CoR. We have continued to emphasize the role of this factor in breast cancer, because it has important implications for the large number of women who have estrogen receptor positive tumors. A major therapeutic problem is the occurrence of resistance to the most widely used estrogen antagonist, tamoxifen or 4-hydroxy tamoxifen. Over the past year we have reached initial understanding of the mechanism of tamoxifen action which works as a repressor in normal breast and in breast tumors, and the molecular basis of the development of tamoxifen resistance. Thus nuclear receptor co-repressor (N-CoR) can bind to the estrogen receptor in the presence of the antagonist (partial agonist) tamoxifen, and while, in turn is dominant to the actions of, activating co-factors that associate with the receptor N-terminus. We believe that these studies will ultimately permit categorization of tamoxifen resistance and new approaches to therapy. The Army Breast Cancer Grant provides the only source of funding for this research, and we believe that we are in a position to provide important contributions to treatment of estrogen receptor positive tumors under the support of this grant.

While we will continue to complete our study of Specific POU domain novel seven transmembrane helix gene expressed in breast, our emphasis will clearly be focused on the estrogen receptor project, because it represents the most important project on which to focus our continued resources under this grant, we believe that, in this way, we will provide the maximum benefit to the area of breast cancer research.

INTRODUCTION

The morphogen retinoic acid is required for development, growth and differentiation (reviewed in 1 and 2). Retinoids, a group of analogs of vitamin A, particularly at high levels, suppress carcinogenesis in various epithelial tissues, including the mammary gland (3-6). We believe that this reflects the actions with a co-repressor that we believe may serve roles in initiation of breast cancer. The hormone effects are mediated by binding to specific nuclear receptors (3) that are members of the steroid/thyroid hormone receptor super family (4-6). This class of proteins functions as ligand dependent transcription factors that mediate the response of the hormone signal by direct control of gene expression. The estrogen and glucocorticoid receptors bind DNA as homodimers, while retinoic acid receptors preferably interact with their cognate DNA response elements as components of heterodimeric complexes (7-20) often involving a partner that our laboratory and others identified to be members of the retinoid X receptor (RXR) family (8-21). Heterodimers of retinoic acid receptor and retinoid X receptor bind with high affinity and activate transcription from response elements consisting of direct repeats, palindrome or inverted palindromic arrangements of a core recognition motif (4, 8-21). The relative orientation and spacing of the core recognition motifs play essential roles in the specificity of the DNA binding and transcriptional activation. While heterodimers of retinoic acid

receptor and retinoid X receptor bind to direct repeats of core motifs spaced by 1, 2 and 5 bp (DR+1, DR+2, and DR+5), an unspaced palindrome binds to an inverted palindromic arrangement of the core motif spaced by 6-8 bp, (e.g. IP+6) reviewed in 22,23).

Recent studies indicate that heterodimeric complexes of retinoic acid receptor and retinoid X receptor molecules exhibit a polarity binding to various DNA elements (24-26), and that this polarity-specific binding may play important roles in cell-specific regulation by retinoic acid receptors. On a direct repeat spaced by 5 bp (DR+5) retinoid X receptor selectively binds to the upstream half-site and retinoic acid receptor binds to the downstream half-site (25,26). When the spacing is reduced by one basepair (DR+4 site) the element becomes a binding site for heterodimers of thyroid hormone receptor and retinoid X receptor. In this case the thyroid hormone receptor is bound to the downstream half-site and the retinoid XD receptor again interacts with the upstream half-site. However, in the case of a direct repeat spaced by 1 bp (DR+1), retinoid X binds on the 3' site (27). The stringency of this polarity-specific binding was further confirmed using specific mutants of retinoid X receptor containing the P-box residues of the glucocorticoid receptor (27). In contrast to the glucocorticoid receptor, retinoic acid receptors are not associated with heat shock proteins in the absence of hormone, but are bound to their response elements and are able to actively repress basic transcription (28,29).

The cellular thyroid hormone receptor, like the viral oncogene erbA of the Avain Erythroblastosis Virus (AEV), V-erbA, represses transcription of target genes in the absence of ligand, with hormone binding resulting in de-repression and activation (31,34,35,45). Evidence has indicated that in most cases ligand-independent repression appears to result from an active repressor function within the ligand binding domain. A ligand-independent repression function could be transferred by the carboxyl-terminal region of the thyroid hormone receptor to heterologous DNA binding domain. Fusion of the C-terminal domains of v-erbA, T_3R, and RAR to the DNA binding domain of the yeast transcription factor GAL4, generated UAS-dependent transcriptional repressor proteins (31). In contrast, the RXR C-terminus fused to the GAL4 DNA binding domain did not mediate transcriptional silencing. Our laboratory and others, were able to show that this repression was mediated by the C-termini of the retinoic acid and thyroid hormone receptors (30-33)c, thus, thyroid hormone and with the polarity of binding on sites with asymmetric core motifs (17, 36-44), dictate whether the receptor exerts either positive control of gene transcription .

The molecular mechanisms responsible for nuclear receptor transcriptional silencing have not been well understood until the past year. Recent studies showed that several nuclear receptors may interact with the basal transcription factors, including TFIIß (47); however, the distal T_3 receptor thyroid hormone C-terminal regions interact with TFIIß, are not sufficient to confer repression. Indeed, the regions in the hinge and N-terminal part of the ligand-binding domain of the thyroid hormone receptor are required for silencing (46,47). Co-transfection experiments suggest that these sequences which do not bind TFIIß can potentially compete for a putative soluble co-repressor molecule (47) and imply that existence of additional interaction factors required for ligand-independent repression. Last year, under this Grant, we isolated and characterized a novel 270 kDa factor (N-CoR) characterized by an interaction domain in the distal C-terminus, and a receptor in the mutant cell and also interacts with retinoid acid receptor, but not with the unliganded estrogen, progesterone, retinoid X, glucocorticoid, or vitamin D receptors. Receptor specific mutations in this region that abolished interactions with the 270 kDa protein also eliminated the ligand-independent repression function of the thyroid hormone receptor, retinoic acid, and because 270 kDa protein can itself function as a repressor, our data suggested that the 270 kDa protein associated with the unliganded, DNA-bound thyroid hormone receptor, and retinoic acid is required for ligand-independent transcriptional repression; we, therefore, termed this protein N-CoR for nuclear receptor co-repressor (51-53).

Several lines of evidence indicate that nuclear receptors must interact with additional factors dependent on a conserved distal C-terminal motif (AF2) to mediate both activation and repression of gene expression (48-52). Biochemical assays have identified 140 and 160 kDa proteins (p140 and p160) (49,48,52) that associate with estrogen, retinoic acid, thyroid hormone, retinoid X, and potentially other nuclear receptors as the most prominent ligand-dependent putative co-activators, binding in an AF2-dependent fashion. In addition, a series of proteins exhibiting ligand-dependent interactions with the C-termini of nuclear receptors that may also function as co-activators have been identified using a yeast two-hybrid screen.

6. BODY

Over the past two years, we have worked to identify the p160 exhibiting ligand-dependent association with DNA-bound thyroid hormone and retinoic acid receptors in the presence of thyroid hormone and to understand how the molecules function. The ligand-binding domains of estrogen receptor and other nuclear receptors, including retinoic acid and thyroid hormone receptors, interact strongly in the cell with a conserved domain in the N-terminus of CBP and p300 in a ligand-dependent manner. Further, the putative co-activator p160 was found to interact independently and specifically with a conserved C-terminal domain in CRIB binding protein CBP and p300. Expression cloning of a family of p160 cDNAs was achieved based on estrogen receptor and CBP interaction. Several independent experimental approaches have suggested a central role of CBP in ligand-dependent activation of RAR and T_3R. One of the p160 factors (NCoA-1) appears to be associated directly with liganded estrogen receptors, while a second (p/CIP) is primarily associated with CBP; yet both are required for estrogen-dependent activation. In addition, the CBP-associated factor harboring intrinsic histone acetylase function p/CAF, is required for nuclear receptor function..

Our data revealed that, in a concentration-dependent fashion, anti-CBP IgG specifically inhibited ligand-dependent activation of transcription units containing retinoic acid response elements, without altering expression of other promoters. (57)

Because CBP and/or its related family members were required for transactivation by retinoic acid and other nuclear receptors, we investigated the possibility that putative nuclear receptor co-activators, p140 or p160, could themselves interact with CBP. A region of 105 amino acids that was sufficient for interactions with p160. In addition, ^{32}P-CBP C-terminus could detect p160 in Far-Western experiments. These cDNAs encoding the putative p160 (nuclear receptor co-activator, NCoA) were obtained by expression cloning based on the criteria that phage plaques exhibit interaction with both CBP C-terminus and liganded nuclear receptors and gene products were identified and full copy cDNA obtained (Figure 3). The first (NCoA-1) encoded a variant forms of the SRC-1 protein, initially reported to have a predicted molecular weight of 115 kDa (54). N-terminally extended variants included forms containing 1465 and 1402 amino acids, with predicted molecular weights of 159 and 152 kDa respectively. We also identified two additional family members (referred to as NCoA-2, and p/CIP). P/CIP proves to be very largely expressed in breast tumor cells. Based on immunodepletion experiments, these p160 factors are the biochemically-identified p160. Microinjection assays confirmed that both NCoA-1 and p/CIP are required for estrogen and retinoic acid receptor-dependent activation events.(55) Nuclear receptors, CBP, NCoA and p/CIP interact with conserved leucine charged residue rich domains (LCD) motifs, apparently ampipathic helices (i.e. hydrophobic on one gene, hydrophalic in the other gene) that are present in all factors known to be recruited to the corepressor complex (57).

N-CoR AND SMRT ARE PART OF A CO-REPRESSOR COMPLEX:

Consistent with a role of N-CoR in mediating the biological effects of estrogen antagonists, decreased N-CoR levels are found to correlate with the acquisition of tamoxifen resistance by human breast cancer cell in nude mouse model.

Recently, N-CoR and SMRT have been found to be components of corepressor complexes (56,58,59) that also include the distinct histone deacetylases HDAC1 (60) and HDAC2/mRPD3 (61), as well as the corepressor mSin3 (62,63). Recent work by our laboratory and others has shown that HDAC2 is recruited to DNA via targeting by diverse site-specific transcription factors (56,58,59,64-71). This led to the hypothesis that histone deacetylation results in a structural rearrangement of chromatin and, by an as yet poorly understood mechanism, transcriptional repression. This model provides a conceptual link between external stimuli, recruitment of corepressor complexes, and resulting alterations of chromatin that modulate gene transcription.

Recently, N-CoR was independently identified as a progesterone receptor-binding protein in the obligatory presence of the antiprogestin RU486 70). Our results suggest that receptors other than the thyroid hormone and retinoic acid receptors may, under some conditions, recruit the N-CoR corepressor complex. Conversely transcriptional activation by nuclear hormone receptors, requires the histone acetylases CBP/p300 (73-73) and p/CAF (61). Thus, the ligand-mediated switch of a nuclear receptor from transcriptional repression to transcriptional activation reflects an exchange of a histone deacetylase-containing corepressor complex for a coactivation complex containing multiple histone acetylase functions (56).

This issue is clinically relevant because the development of inhibitory ligands for the nuclear receptors, that seemingly do not cause the switch in complexes, has yielded important therapeutic treatments, among them the use of tamoxifen for endocrine therapy of breast cancer. The tamoxifen-related compounds, including trans-hydroxytamoxifen (TOT) are thought to inhibit estradiol-dependent transactivation by competitive binding to the estrogen receptor (74,75). However, in certain tissues such as uterus and bone, and after long term treatment in patients with breast cancer, tamoxifen exhibits partial agonistic activity thought to be mediated by the constitutively active AF-1 domain of the estrogen receptor, although the mechanism by which tamoxifen exerts differential effects in various tissues has remained elusive (76). Substances which raise intracellular cAMP levels or stimulate the ras/MAP kinase pathway can also cause the estrogen receptor to activate in the absence of its activating ligand (77-83).

Over the past year, we have demonstrated that diverse strategies are used by the breast all in regulating the association of specific N-CoR or SMRT-containing complexes with nuclear receptors, which includes in addition to the DNA site, the nature of the ligand, the levels of N-CoR/SMRT, and the action of several protein kinase-dependent signaling cascades. These diverse regulatory events alter the association of the receptor with corepressors and coactivators, and coordinately dictate whether these nuclear receptors will repress or activate the transcription of target genes.

The regulation of the corepressor binding appears to be biologically important for diverse functions; for example, retinoic acid receptors prove to be constitutively active in the absence of N-CoR, and the absence of either N-CoR or SMRT converts steroid receptor antagonists to functional agonists by allowing the receptor N-terminus to recruit the identical CBP/ p/CIP complex required for activation by the liganded estrogen receptor C-terminus. These regulatory events dictate the nature of the ligand response in normal and tumor cell types, and present several new approaches to problems of resistance in antagonists-treated, receptor positive breast cancers.

7. CONCLUSIONS

Modulation of the association of receptors with corepressor complex

Recent evidence has suggested that nuclear receptor function is dependent upon the recruitment of specific coactivator and corepressor complexes. In this manuscript, we present evidence that regulation of the corepressor complex is critical for normal homeostasis and that it actually serves to impose ligand-dependence on those nuclear receptors that bind N-CoR constitutively. Thus, when the binding of N-CoR to the unliganded retinoic acid receptor is prevented, the receptor becomes capable of functioning as a constitutive activator. An antiprogestin and the Class I antiestrogens (tamoxifen-like) induce recruitment of the N-CoR corepressor complex to receptors that, unlike thyroid hormone and retinoic acid receptors, do not bind effectively to corepressors in the unliganded state (51). Even pure antagonists (e.g. LG 629) of the retinoic acid receptor fail to entirely inhibit activation in the absence of N-CoR binding. Similarly, blocking receptor association with N-CoR converts the anti-estrogen TOT into an agonist, suggesting that either a decreasing level of N-CoR, or inhibition of corepressor binding to the receptor, might account for the ability of TOT to induce activation in specific cell types and we provide evidence that both types of regulation do occur. Therefore, a critical biological role of the N-CoR corepressor complex is to suppress constitutive activation and thus impart ligand dependence on the many developmental and homeostatic events controlled by specific nuclear receptors.

Our studies, in concert with reports that overexpression of N-CoR/SMRT opposes the effects of coactivators (SRC-1/NCoA-1 in particular) that allowed antagonistic ligands to act as partial agonists, indicate that the cell type-specific activation effects of tamoxifen derivatives are mediated by the receptor N-terminal (AF-1) domain and are normally blocked by the association of N-CoR with the receptor C-terminus. Thus activation function of the N-terminus appears itself to be dependent upon SRC-/NCoA-1 and the p/CIP/CBP complex. While there are weak *in vitro* interactions between the estrogen receptor N-terminus and p/CIP, as well as SRC-1/NCoA-1, it is likely that these coactivators participate in either cooperative interactions with the receptor C-terminus and/or that an additional N-terminus associated bridging factor(s) stabilizes the binding of the coactivator complex. Indeed, stimulation of cAMP-dependent and MAPK signaling pathways in cells appears to increase the interactions between the nuclear receptor AF-1 domain and components of the p/CIP/CBP complex, in parallel to facilitating the dismissal of N-CoR from the receptor AF-2 domain. We hypothesize that N-CoR or SMRT complexes on the antagonist-bound receptor C-terminus weakly interact with the constitutive receptor N-terminus and influence the association of coactivators. We propose that, two independent activation domains in the same receptor recruit the identical coactivator complex, or a complex containing at least a subset of the factors required in ligand-induced response, and this accounts for the instances of reported synergy between those domains (84,85).

These observations support the proposal that the nature of the transcriptional response to ligand depends on the highly regulated ability of nuclear receptors to accurately shift between a coactivator complex with histone acetylase activity and a corepressor complex containing histone deacetylase activity and provides a model system for other classes of transcription factors.

Regulation of corepressor complexes in vivo

The biochemical data presented in this manuscript predict that any decrease in levels of N-CoR or in the affinity of the receptor for the corepressor could cause a shift in tamoxifen from antagonist to agonist, with clear implication in regard to certain pathological conditions and the use of receptor antagonists in treatment of cancers. Indeed, in mouse models of tamoxifen resistance, we observe a statistically significant correlation between decreased levels of N-CoR and

the transition of MCF7-derived tumors from tamoxifen-sensitivity to tamoxifen-resistance. Additionally, many breast tumors eventually develop high levels of tyrosine kinase receptors or of intracellular cAMP (86,87) which, according to our data, would be predicted to favor the conversion of tamoxifen to agonist function by causing release of the corepressor complex. In contrast, as HepG2 cells seem to contain a normal complement of N-CoR alone, we speculate that a signaling pathway which decreases recruitment of the N-CoR component is misregulated in these cells, causing failure of TOT to act as an antagonist in the cell line. As stimulation of protein kinase A and MAP kinase pathways in the presence of estradiol results in synergistic activation of estrogen receptor in ell-type specific manner (88), these pathways could also act to amplify the agonist activity of tamoxifen even at normal levels of N-CoR, promoting tumor growth. A switch in preference for binding the coactivator or corepressor complexes provides a mechanism by which signal transduction events initiated at the cell membrane can influence diverse nuclear receptors.

PLANS FOR NEXT YEAR

As outlined in our Plans for least year's Progress report, this year we plan:

Task I To continue to generate and analyze animals null for the BrR receptor factor genomic loci.

Task II (Continuation from Year 2 Progress Report) Further investigate the regulation of the nuclear receptor co-repressor, N-CoR, in breast tumors.

Task III (Continuation from Year 1 Progress Report) To further study the recently identified p/CIP and the role of its gene amplification in breast cancer.

Task IV (Initiated from Year 2) To further link the co-repressor and co-activator to drug-resistant breast cancer. We will further investigate the model that inhibitors (tamoxifen) used in clinical breast cancer causing estrogen receptor to bind the nuclear receptor co-receptor, and are controlled both by regulation of levels of NCoR and by specific signal transduction pathways, in clinical cases of tamoxifen resistance.

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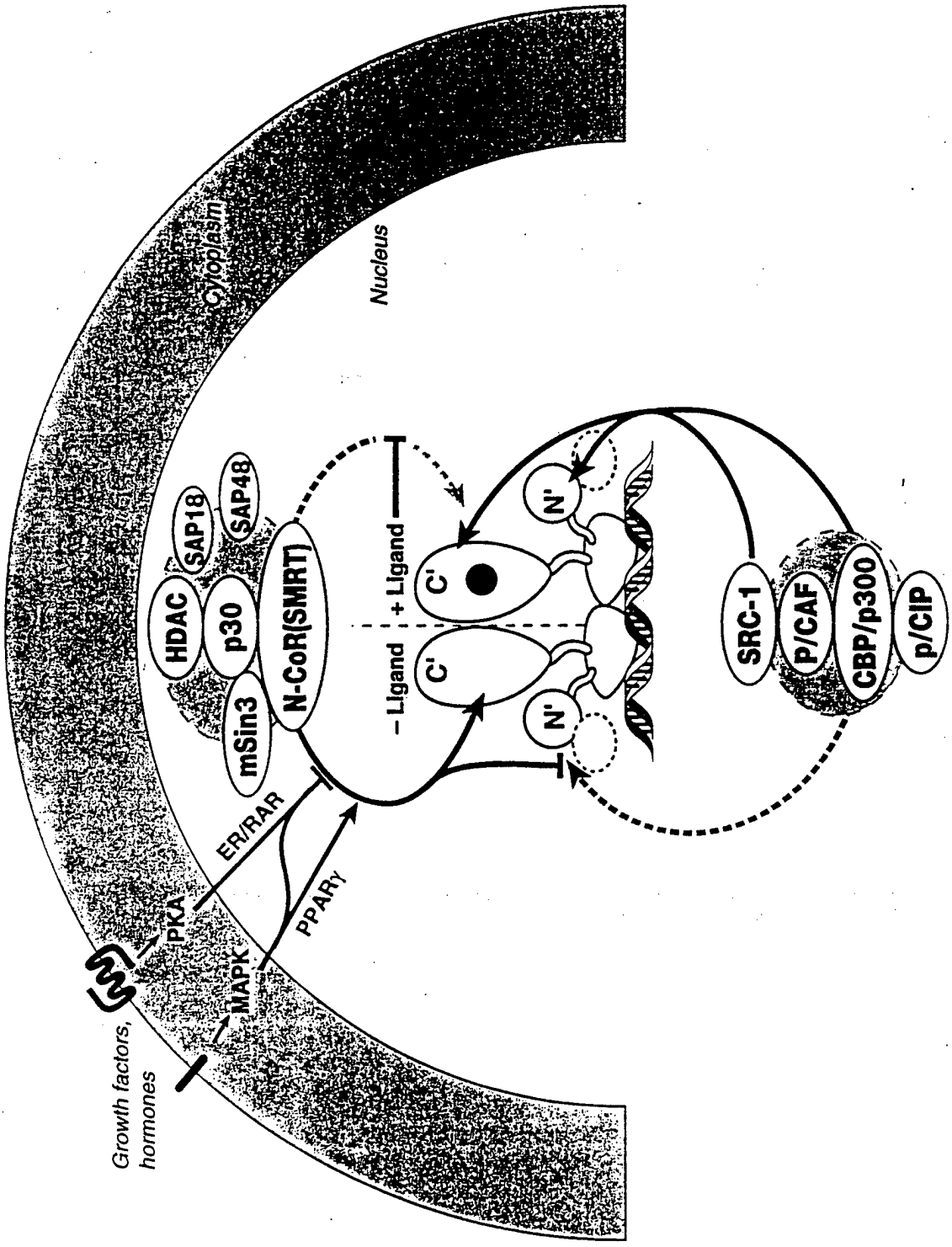
9. APPENDIX

Figure 1: Model of regulation of specific corepressor complexes with nuclear receptors, and inhibition of the N-terminal activation function. Both ligands and external signaling pathways regulate the association of specific corepressor and coactivator complexes with nuclear receptors. At least one member of a receptor homo- or hetero-dimer binds strongly, in the absence of ligand, to either the N-CoR or SMRT-containing corepressor complex which localizes histone deacetylase activity to the promoter. This complex also simultaneously inhibits a constitutive N-terminal activation domain of the receptor. In the absence of the corepressor complex, and dependent at least in part on an N-terminal domain, receptors can exhibit constructive activity. The corepressor complex is dismissed by agonist ligands which simultaneously recruit an acetylase-containing coactivator complex that interacts with both the C-terminal AF-2 and the AF-1 activation domains in the receptor. Phosphorylation-dependent signaling pathways, initiated at the cell membrane, influence receptor activity by inhibiting the recruitment of the corepressor complex to steroid (ER/PR) and retinoid (RAR) receptors or, conversely, by stimulating its recruitment of peroxisome proliferator-activated receptor γ (PPARγ).


Growth factors, hormones
PKA
MAPK
ER/RAR
PPARγ
Cytoplasm
Nucleus
HDAC
SAP18
SAP48
p30
mSin3
N-CoR(SMRT)
– Ligand
+ Ligand
C'
N'
SRC-1
P/CAF
CBP/p300
p/CIP